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Apoptosis induction by γ-tocotrienol in human hepatoma Hep3B cells

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Abstract

We evaluated the antitumor activity of tocotrienol (T3) on human hepatoma Hep3B cells. At first, we examined the effect of T3 on the proliferation of human hepatoma Hep3B cells and found that γ-T3 inhibited cell proliferation at lower concentrations and shorter treatment times than α-T3. Then, we examined the effect of γ-T3 apoptosis induction and found that γ-T3 induced poly (ADP-ribose) polymerase (PARP) cleavage and stimulated a rise in caspase-3 activity. In addition, γ-T3 stimulated a rise in caspase-8 and caspase-9 activities. We also found that γ-T3-induced apoptotic cell death was accompanied by up-regulation of Bax and a rise in the fragments of Bid and caspase-8. These data indicate that γ-T3 induced apoptosis in Hep3B cells and that caspase-8 and caspase-9 were involved in apoptosis induction. Moreover, these results suggest that Bax and Bid regulated apoptosis induction by γ-T3.

Keywords: Tocotrienol; Human hepatoma Hep3B cells; Apoptosis

1. Introduction

Tocotrienol (T3) has a basic chemical structure with a long unsaturated phytyl chain attached to the 1-position on the chroman ring. In addition, T3 homologues differ from each other by the number of methyl groups in the chroman ring. T3 has been reported to have various activities, such as antioxidative, hypocholesterolemic and immunoregulatory activities [1,2]. It has also been reported to inhibit the proliferation of rat hepatoma dRLh-84 cells, normal mouse mammary epithelial cells, human breast carcinoma MCF-7, human leukemia HL-60 and murine B16 melanoma cells in vitro [3–6], and skin cancer cells in vivo [6]. In addition, it has been reported that tumor marker enzyme activities in rats with chemically induced cancer are moderated by α-tocopherol and γ-T3 supplementation [7].

Furthermore, T3 has been reported to induce apoptosis [4]. When apoptosis is induced in mammalian cells, various types of caspases are activated. These caspases are considered to be executive factors for apoptosis. It has been believed that the activation of caspase is induced through two pathways. In the first pathway, binding of cytochrome *c* released from mitochondria to Apf-1 participates in the activation of caspase-9. In the second pathway, binding of death ligand to death receptors such as Fas and TNF receptor participates in the activation of caspase-8. Caspase-8 and caspase-9 activate caspase-3 [8–12], and activated caspase-3 cleaves poly (ADP-ribose) polymerase (PARP) [13]. The cleavage of PARP is used as an indicator of apoptosis.

On the other hand, the Bcl-2 family of proteins exert anti-apoptotic or pro-apoptotic activity through the control of mitochondrial membrane permeability during apoptosis [14]. Anti-apoptotic factors such as Bcl-2 avoid cell death by stabilizing the mitochondrial membrane, while pro-apoptotic ones such as Bax and Bid induce cell death by enhancing mitochondrial membrane permeability, which leads to the release of cytochrome *c* [15–17]. In addition, Bid is cleavaged by caspase-8, and its fragmentation enhances the release of cytochrome *c* from mitochondria [18–20]. In this study, we examined the mechanism of apoptosis induction by γ-T3 in human hepatoma Hep3B cells.

2. Materials and methods

2.1. Materials

T3 derivatives such as α-, γ- and δ-T3 were kindly presented by Eisai (Tokyo, Japan). The BCA protein assay kit was purchased from PIERCE (Rockford, IL). The

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caspase-3 substrate Ac-DEVD-MCA and the caspase-9 substrate Ac-LEHD-MCA were purchased from Peptide Co. (Osaka, Japan), respectively. The caspase-8 substrate Ac-IETD-AMC was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA).

2.2. Cells and cell culture

Human hepatoma Hep3B cells were purchased from Japan Cancer Research Resources Bank (Tokyo, Japan). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin and 5% fetal bovine serum (FBS). The cells were cultured in 5% FBS/DMEM with α-, γ- or δ-T3 for the appropriate periods described in each experimental method. As a vehicle, 0.1% of ethanol was added to each DMEM containing none groups. Then, attached cells were trypsinized with 0.2% trypsin containing 0.025% ethylene diamide tetraacetic acid (EDTA) dissolved in phosphate buffered saline (PBS, pH 7.4), and the number of viable cells was counted using the trypan blue exclusion method.

2.3. Immunoblot analysis for PARP cleavage

Hep3B cells were treated with 25 or 50 μM γ-T3 for 24 h. Then, adherent cells were collected with trypsinization. Detached cells were also collected. These cells were washed with PBS three times and then lysed in PBS containing 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1% Tergitol type NP-40, 5 μg/ml phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinine. After the determination of the protein content using the BCA protein assay kit, the lysate was applied to the electrophoresis using 7.5% SDS-polyacrylamide gel, and the gel was transferred to a PROTRAN nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). After blocking with Block Ace (Snow Brand, Sapporo, Japan) for 1 h at 37°C, the membrane was incubated with a 1000-fold diluted rabbit antihuman PARP antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 37°C. Finally, the membrane was reacted with a 20,000-fold diluted peroxidase-conjugated goat antirabbit IgG (ICN Pharmaceuticals, Aurora, OH) for 45 min at 25°C. The membrane was washed three times with PBS containing 0.05% Tween-20 every antibody binding reaction. Detection of PARP protein was performed using the ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, England).

2.4. Determination of caspase activity

Hep3B cells were treated with 50 μM γ-T3 for 18 or 24 h for determination of caspase-3, caspase-8 and caspase-9 activities. Both adherent and detached cells were collected and then washed once with PBS. The cell pellet was suspended in the extraction buffer containing 25 mM HEPES, 5 mM EDTA, 5 mM EGTA, 1 mM $MgCl_2$, 2 μg/ml aprotinine, 5 μg/ml PMSF and 100 μM dithiothreitol, and then kept on ice for 30 min. After sonication for 30 s, the cell lysate was centrifuged for 20 min at 15,000×*g* and 4°C. The protein content of the supernatant was measured using the BCA protein assay kit before analyses of caspase-3, caspase-8 and caspase-9 activities. The supernatant was mixed with the assay buffer containing 25 mM HEPES, 10% sucrose, 0.1% CHAPS and 100 μM dithiothreitol, and then incubated for 10 min at 37°C. The reaction was started with 10 μM Ac-DEVD-MCA for caspase-3, Ac-IETD-AMC for caspase-8 and Ac-LEHD-MCA for caspase-9, and the amount of MCA was determined using RF-1500 spectrofluorophotometer (Shimadzu, Kyoto, Japan).

2.5. Western blotting analyses for Bax, Bcl-2, Bid and caspase-8

Hep3B cells were treated with 25 or 50 μM γ-T3 for 18 h. Then, cells were collected, washed three times with PBS and lysed with PBS containing 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 1% Tergitol type NP-40, 5 μg/ml PMSF, 2 μg/ml aprotinine. After the determination of the protein content using the BCA protein assay kit, the lysate was applied to the electrophoresis using a 15% SDS-polyacrylamide gel, and the gel was transferred to a PROTRAN nitrocellulose membrane. After blocking with bovine serum albumin (Roche, Penzberg, Germany) for 1 h at 37°C, the membrane was incubated with a 1000-fold diluted rabbit antihuman Bax, Bcl-2 or Bid antibody (Santa Cruz Biotechnology) for 1 h at 37°C. Finally, the membrane was treated with a 20,000-fold diluted peroxidase-conjugated goat antirabbit IgG for 45 min at 25°C. The membrane was washed three times with PBS containing 0.1% Tween-20 every antibody binding reaction. Detection of protein band was performed using the ECL kit.

For caspase-8 expression, the lysate was applied to the electrophoresis using a 10% SDS-polyacrylamide gel and then transferred to a PROTRAN nitrocellulose membrane. After blocking with nonfat milk (Snow Brand) for 1 h at 37°C, the membrane was incubated with 1000-fold diluted rabbit antihuman caspase-8 antibody (Medical and Biological Laboratories, Nagoya, Japan) for 1 h at 37°C. Finally, the membrane was treated with a 3000-fold diluted peroxidase-conjugated goat antimouse IgG (Zymed Laboratories, San Francisco, CA) for 45 min at 25°C. The membrane was washed three times with PBS containing 0.1% Tween-20 every antibody binding reaction. Detection of the PARP protein was performed using the ECL kit. All experiments were replicated at least two times.

3. Results

3.1. Effect of tocotrienols on the proliferation of Hep3B cells

Fig. 1 shows the effect of α-, γ- and δ-T3 on the proliferation of Hep3B cells cultured for 72 h. In the cells, α-T3 exerted a weak inhibitory activity only at 75 μM. On the other hand, γ-T3 exerted a strong killing activity over

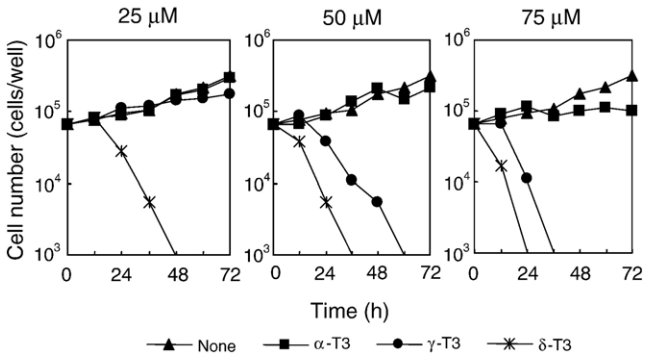

Fig. 1. Growth curves of Hep3B cells in the presence of α-T3, γ-T3 and δ-T3. These cells were inoculated at 5×10^4 cells/well and cultivated in DMEM supplemented with 5% FBS for 0, 12, 24, 36, 48, 60 and 72 h with 25, 50 and 75 μM α-T3, γ-T3 and δ-T3 ($n=1$).

50 μM from 24 h after T3 addition. The killing activity of δ-T3 was observed from 25 μM much more strongly than that of γ-T3. The killing activity of δ-T3 was observed from 24 h after T3 addition at 25 μM and 12 h after the addition at 50 and 75 μM. These results indicate that the strength of the antiproliferative effect is in the order of δ-T3>γ-T3>α-T3. δ-T3 possesses a higher antiproliferative effect than γ-T3. However, δ-T3 content in foodstuffs is very low compared with α-T3 or γ-T3. Palm oil contains the highest level of γ-T3, but γ-T3 level is lower than that of α- or γ-T3 in palm oil [21]. Therefore, we studied the killing mechanism of γ-T3 thereafter. This experiment was repeated to ensure its reproducibility.

3.2. Effect of γ-T3 treatment on PARP fragmentation and caspase activity

Fig. 2 shows the effect of γ-T3 on PARP cleavage in Hep3B cells. PARP is a 116-kDa protein in its intact form and is decomposed to an 85-kDa fragment. When the cells were cultured with 25 μM γ-T3 for 24 h, a 116-kDa band of PARP was recognized, but not an 85-kDa fragment. On the other hand, both the 116- and 85-kDa bands were detected in the cells treated with 50 μM γ-T3. This experiment was repeated to ensure its reproducibility. These results suggest that the 50 μM γ-T3 treatment induced apoptosis in the cells.

Thus, the effect of vitamin E homologues on caspase-3, caspase-8 and caspase-9 activities in Hep3B cells was examined (Table 1). When the cells were treated with 50 μM γ-T3, caspase-3 activity was about six times higher than that of untreated control cells at 18 h and 21 times higher at 24 h.

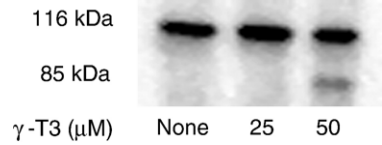

Fig. 2. Cleavage of PARP protein by γ-T3 in Hep3B cells. Cleavage of PARP protein was examined after the cells were inoculated at 5×10^4 cells/well and cultivated in DMEM supplemented with 5% FBS for 24 h with γ-T3 at 25 or 50 μM.

Table 1
Effect of γ-T3 on caspase-3, caspase-8 and caspase-9 activities in Hep3B cells

	18 h		24 h	
	None	γ-T3	None	γ-T3
	Released MCA level ($\times10^{-7}$ pmol/min per mg protein)			
Caspase-3	0.90	5.51	1.15	23.9
Caspase-8	5.20	18.0	7.71	8.17
Caspase-9	4.20	11.5	2.21	4.46

Caspase-3, caspase-8 and caspase-9 activities were measured after the cells were inoculated at 5×10^4 cells/well and cultivated in DMEM supplemented with 5% FBS for 18 or 24 h with γ-T3 at 50 μM. The amount of released MCA levels is treated as the indicator of each caspase activity ($n=1$).

The increases in caspase-8 and caspase-9 activities were milder than that of caspase-3. Caspase-8 activity of the cells treated with γ-T3 was about 3.5 times higher than that of control cells at 18 h, but comparable at 24 h. Caspase-9 activity of T3-treated cells was about three times higher than that of control cells at 18 h and about two times higher at 24 h. These experiments were repeated to ensure their reproducibility. These results suggest that caspase-8 and caspase-9 are activated earlier than caspase-3, and the activities of the former are involved in the induction of apoptosis by γ-T3.

3.3. Effect of γ-T3 treatment on the expression of the Bcl-2 family of proteins

Then, we examined the effect of γ-T3 treatment on the expression of the Bcl-2 family of proteins as well as caspase-8 protein, to clarify the mechanism of γ-T3-induced apoptosis. As shown in Fig. 3, γ-T3 treatment affected the expression of Bax, Bcl-2, Bid and caspase-8 proteins. When the cells were treated with 25 μM γ-T3 for 18 h, the expression level of Bax and Bcl-2 was not changed in comparison with the control. On the other hand, Bax protein level was higher in the cells treated with 50 μM γ-T3 than that of the control or 25 μM γ-T3-treated cells. In Bcl-2 expression, significant effect was not induced by γ-T3 treatment. Bid protein level was not changed in the cells

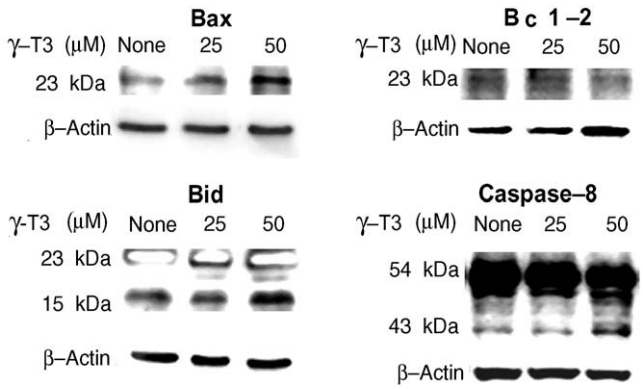

Fig. 3. Western immunoblot analyses of Bax, Bcl-2, Bid and caspase-8 by γ-T3 in Hep3B cells. Bax, Bcl-2, Bid and caspase-8 protein were examined after the cells were inoculated at 5×104 cells/well and cultivated in DMEM supplemented with 5% FBS for 18 h with γ-T3 at 25 or 50 μM.

treated with 25 μM γ-T3, but the level of the 15-kDa fragment in the cells was higher than that of the control or 25 μM γ-T3-treated cells. Similarly, changes in caspase-8 protein level were observed only in the cells treated with 50 μM γ-T3. The level of the major 54-kDa band was lower at 50 μM and that of the 15-kDa fragment higher than that at 0 and 25 μM. These experiments were repeated to ensure their reproducibility. These results suggest that the Bcl-2 family of proteins participate in the regulation of apoptosis induction by γ-T3.

4. Discussion

Anticancer activity has been reported in various food components, such as tea catechin [22], capsaicin [23], polyunsaturated fatty acids [24,25] and dietary fibers [26]. It has also been reported that lipophilic vitamins such as vitamins A, D and K have antitumor effect, but the mechanism of expression remains unknown [27–29].

Among vitamin E homologues, T3 has been reported to inhibit the proliferation of rat hepatoma dRLh-84 cells, normal mouse mammary epithelial cells, human breast carcinoma MCF-7, human leukemia HL-60 and murine B16 melanoma cells [3–6]. In addition, it has been reported that they inhibit the development of skin cancer in vivo [6]. In this study, Hep3B cells were treated with 25, 50 or 75 μM of T3. Previously, we showed that high level of T3s exists in the liver of rats on the marrow of T3 feeding [2,30]. We used T3 concentrations according to this information. We showed here that γ-T3 derivatives exert a strong cytotoxic effect against human hepatoma Hep3B cells, in a structure-dependent manner. In the cells, killing activity was strongly detected in the order of δ-, γ- and α-T3. This suggests that the strength of the T3 derivatives is dependent on the structure of the chroman ring.

Cell death is classified into necrosis and apoptosis. In the induction of apoptosis, activation of caspases plays an important role [8,9]. Among them, caspase-8 or caspase-9 was first activated and then caspase-3 is activated. When caspase-3 is activated, PARP is cleaved. Thus, cleavage of PARP is used as an indicator of apoptosis [13]. We showed here that γ-T3 induced fragmentation of PARP as well as increase in caspase-3 activity in Hep3B cells, suggesting that γ-T3 induced apoptosis against Hep3B cells. A similar increase in caspase-3 activity has been reported in dRLh-84 cells and highly malignant +SA mouse mammary epithelial cells in the presence of γ-T3 [3,31,32]. In addition, it has been reported that tocotrienol-rich fraction (TRF) of palm oil enhances caspase-3 activity in human colon carcinoma RKO cells and highly malignant +SA mouse mammary epithelial cells [31,33]. However, fragmentation of PARP was not detected in the induction by γ-T3 in human breast cancer MDA-MB-231 cells despite the obvious induction of apoptosis [34]. This suggests that the participation of caspase-3 activation in apoptosis is cell line-dependent.

In this study, the increase in both caspase-8 and caspase-9 activities was detected in Hep3B cells treated with γ-T3. These results suggest that the expression of apoptosis induced by γ-T3 in Hep3B cells is involved in the induction of both caspase-8 and caspase-9 activities. We have reported that the rise in caspase-8 activity was detected in dRLh-84 cells [3]. On the other hand, the participation of caspase-8 has been reported in highly malignant +SA mouse mammary epithelial cells (+SA) treated with γ-T3, but not caspase-9 [31,32]. These results suggest that the participation of caspase-8 and caspase-9 is also cell line-dependent.

The Bcl-2 family of proteins has been reported to regulate apoptosis by controlling the mitochondrial membrane permeability [14]. Bcl-2 suppresses apoptosis by stabilizing the mitochondrial membrane, while Bax and Bid induce apoptosis by enhancing mitochondrial membrane permeability, which leads to the release of cytochrome *c* from mitochondria [15–17]. In addition, Bid is cleavaged by caspase-8, and the fragmentation enhances the release of cytochrome *c* from mitochondria [18–20]. We showed here that Bax protein expression is up-regulated in Hep3B cells treated with γ-T3. Besides, expression of Bid fragment and caspase-8 was increased by γ-T3 treatment. Similarly, alteration of Bax/Bcl-2 ratio in favor of apoptosis has been reported in RKO cells treated with TRF [33]. However, Bcl-2 expression was not significantly affected by γ-T3 treatment.

These results indicate that γ-T3 induced apoptosis in Hep3B cells and that caspase-8 and caspase-9 were involved in apoptosis induction. Furthermore, these results suggest that Bax and Bid participate in the regulation of apoptosis induction by γ-T3.

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